Biological and Molecular Characterization of Two Distinct Tomato Strains of *Cucumber mosaic virus* Based on Complete RNA-3 Genome and Subgroup Specific Diagnosis

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Received: 16 June 2011 / Accepted: 16 August 2011 / Published online: 14 September 2011
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Abstract *Cucumber mosaic virus* (CMV) is one of the most important viral pathogen infecting several plant species in India. Five isolates of CMV obtained from cucumber, muskmelon, tobacco and tomato from distinct geographical locations in India were analysed based on host-reactions and genome sequence. The majority of the isolates were very similar and only two isolates, Tfr-In and Tss-In showed distinct symptoms in tomato and high sequence diversity (77.8%) in coat protein (CP) gene. Tfr-In was isolated from tomato fruit showing grey patches in Aurangabad and Tss-In from tomato plant showing shoe-string symptoms in New Delhi. The RNA-3 genomes of Tfr-In (2,214 nt; JF279606), shared only 70.3% nucleotide sequence identity with Tss-In (2,178 nt; JF279605). The complete RNA-3 genome of Tss-In and Tfr-In were compared with that of 65 CMV isolates reported from various plants of the world, which formed four distinct subclades-IA, -IB, -IC and -II. The Tfr-In isolate clustered with the CMV subgroup-IB and Tss-In with the subgroup-II. The comparison of the RNA-3 sequence of both the isolates revealed maximum heterogeneity in the intergenic region (IR). Reverse transcriptase polymerase chain reaction (RT-PCR) based detection of CMV subgroup-I and -II was developed designing primers from flanking IR region. The specificity of the RT-PCR detection was confirmed using Tfr-In and Tss-In representing subgroup-I and -II and

validated with field samples of tomato, cucurbits and chilli. This is the first report of complete RNA-3 of subgroup-IB CMV causing grey patches in tomato fruit and subgroup-II CMV causing shoe-string symptoms in tomato in India. The present and previous studies together showed that tomato in India was affected by multiple strains of CMV.

Keywords *Cucumber mosaic virus* · Tomato · CMV subgroups · Shoe-string · Grey-patch · RT-PCR · RNA-3 genome

Introduction

Cucumber mosaic virus (CMV) is a single-stranded positive sense RNA plant virus belonging to the genus *Cucumovirus* of family *Bromoviridae*. It is distributed worldwide and has a very broad host range [20] of about 1,200 species causing severe damage in some of the most economically important species under Solanaceae and Cucurbitaceae families. CMV has tripartite genome consisting of three RNAs (RNA-1, RNA-2 and RNA-3), RNA-1 and RNA-2 encodes protein required for replication of the virus [6, 13]. RNA-3 contains two genes, 3a encoding cell to cell movement protein (MP) and 3b encoding coat protein (CP) [21]. Numerous isolates/strains of CMV have been reported from several countries. CMV isolates are grouped into two main subgroups-I and -II based on their biological, serological and molecular properties [13]. Based on the diversity of CP gene and 5' untranslated (UTR) sequence, subgroup-I has been further divided into two groups, IA and IB [12, 20]. Subgroups-IA and -IB shares a close sequence relationship of 92–95% identity, while subgroups-II are quite distantly related to subgroup-I with only 75% sequence identity [19].

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In India, CMV is an economically important and widely occurring plant virus and has been reported from many different host plants such as cucumber [4], black pepper [5], *Amaranthus*, *Datura* [28], *Rauvolfia serpentine*, *Jatropha curcas* [16, 17], Egyptian henbane [23], gladiolus [15], tomato [29], geranium [32], banana [1]. Tomato, an important vegetable crop is cultivated throughout India. CMV is one of the important viral pathogen of tomato where it induces various symptoms—necrosis, mottling, mosaic, narrowing or shoe-string of leaves and stunting of plants [9, 14, 33]. The molecular diversity among CMV isolates in India and elsewhere have been studied mainly based on CP gene sequence. However, the recent database showed availability of 65 full-length RNA-3 sequences of CMV isolates from different countries including 10 from India.

In the present study, five isolates of CMV from different crops in distant geographical locations in India were studied based on host reactions and sequence diversities in CP gene. Two tomato isolates with distinct symptom phenotypes and high sequence diversity in CP gene were identified and complete RNA-3 genome sequence was generated. Phylogenetic relationships of these two isolates with the other CMV isolates occurring worldwide was established. The comparison of complete RNA-3 of the two isolates showed distinct sequence heterogeneity at the end of MP and beginning of CP open reading frame (ORF),

which were utilised to develop reverse transcriptase polymerase chain reaction (RT-PCR) based detection of CMV subgroup-I and -II.

Materials and Methods

Virus Isolates and Transmission

CMV isolates were collected from different hosts and places in India (Table 1). Presence of CMV in these samples was confirmed by transmission electron microscope and enzyme linked immunosorbent assay (ELISA) using polyclonal antibody to CMV. All the isolates were mechanically inoculated to cucumber, *Nicotiana glutinosa* and tomato cv. Pusa Ruby using 0.1 M phosphate buffer, pH 7.2 containing 0.15% sodium sulphite under greenhouse conditions. Symptoms were recorded 5 weeks post inoculation. Transmission was confirmed by RT-PCR using CP based primers, BM05F and BM06R (Table 2).

RNA Extraction

The double stranded RNA (dsRNA) was isolated with slight modifications of the method described by Dodds et al. [7]. Briefly, about 0.2 g of leaf tissue was ground with

Table 1 Isolates of CMV used in the study

S. no.	Isolate	Sample	Place	Collection year	Field symptom	Symptoms under greenhouse conditions ^a		
						Tomato	Cucumber	<i>N. glutinosa</i>
1	Tfr-In	Tomato fruit	Aurangabad	2005	Grey patches on tomato fruit	cs	m	mb
2	Ban-In	Tobacco leaf	Bangalore	2007	Mottling	mt	m	mb
3	Pun-In	Cucumber leaf	Pune	2007	Mosaic	mt	mt	mb
4	Bal-In	Muskmelon leaf	Ballabgarh	2008	Mottling	cs	mt	mb
5	Tss-In	Tomato leaf	New Delhi	2008	Shoe-string	mt, ss	–	mb

^a Symptoms developed following sap inoculation (*mb* mosaic blistering, *m* mosaic, *mt* mottling, *ss* shoe-string, *cs* chlorotic spots)

Table 2 Primers used for the amplification of CMV isolates

Primer	Sequence (5'–3')	Expected product (bp)	Annealing temperature (°C)	Used for amplification of
BM05F	AGTCGAGTCATGGACAAATC	660	45	CP
BM06R	TTAGACTGGGAGACCCC			Subgroup-I and -II
BM35F	GTAATCTTACCACTGTGTGT	960	52	5' UTR + MP
BM38R	CTAAAGACCGTTAACCACC			
BM05F	AGTCGAGTCATGGACAAATC	980	50	CP + 3' UTR
BM36R	TGGTCTCCTTTTGGAGACC			
BM51F	AACAATAGCTTCAGATCGCA	376	54	IR
BM52R	ACTAGCATTGGGAGATCCA			Subgroup-II
BM53F	CCGAAACCTTTAGTCCGC	386	54	IR
BM54R	CCGGCACTGGTTGATTCA			Subgroup-I

liquid nitrogen and RNA was isolated using phenol, chloroform–isoamyl alcohol and CF-11. The dsRNA bound to CF-11 was released in 1X STE and isolated by alcohol precipitation. Finally, the dsRNA was dissolved in 25 µl of RNase free distilled water and stored at -80°C .

RT-PCR and Cloning of RNA-3

RT-PCR for all the isolates was carried out by using CP gene specific primers of CMV, BM05F and BM06R (Table 2) and the amplified fragments were cloned in pGEM T-Easy vector (Promega Madison, WI, USA) and sequenced. Two distinct isolates, Tss-In and Tfr-In showing considerable sequence diversity in CP gene were selected for cloning of complete RNA-3 genome. The complete RNA-3 genome was cloned in three segments: the 5' segment containing 5'-UTR and MP ORF and 3' segment containing 3'-UTR and CP ORF were amplified with the primers, BM35F & BM38R and BM05F & BM36R (Table 2; Fig. 1), respectively, designed based on the comparison of the sequences of CMV isolates available in the database. The remaining intergenic region (IR) between 5' and 3' fragments was amplified with the primers BM51F and BM52R for Tss-In isolate and BM53F and BM54R for Tfr-In isolate, which were obtained from the specific sequence of 5' and 3' segments (Table 2; Fig. 1). Genome amplification was carried out by two steps RT-PCR using RevertAidTM First Strand cDNA Synthesis Kits (MBI Fermentas, USA) and Platinum Taq DNA polymerase (Invitrogen, USA), in a Biometra T Personal thermocycler with the following temperature cycle: cDNA synthesis at 37°C for 1 h, followed by PCR: one cycle at 94°C for 2 min, 40 cycles at 94°C for 30 s, $45\text{--}54^{\circ}\text{C}$ (Table 2) for 1 min and 72°C for 1 min and final extension at 72°C for 10 min. The RT-PCR amplicons were purified by using Wizard SV gel and PCR clean up Kit (Promega Madison, WI, USA) and ligated into a linear pGEM T-Easy vector by using T4 DNA ligase (Promega Madison, WI, USA). Cells of *E. coli* strain JM109 were transformed with the ligation products [24] and the recombinant clones were identified by PCR and restriction digestion and sequenced in ABI 3130 Genetic Analyzer at Chromous Biotech Pvt Ltd, Bangalore.

Sequence Analysis

The complete sequences of RNA-3 genome for both the isolates were Tfr-In and Tss-In assembled by aligning on overlapping regions using BioEdit software (<http://www.mbio.ncsu.edu/BioEdit/BioEdit.html>). The RNA-3 of Tfr-In and Tss-In isolates were compared with that of 65 isolates of CMV retrieved from the GenBank [<http://www.ncbi.nlm.nih.gov/Genbank/index.html>]. The sequence data, accession, strain/isolate, country of origin, and year of collection were presented in the Table 3. Multiple alignment and sequence identity matrix of the sequences were performed using ClustalW programme. Phylogenetic analysis was conducted in MEGA4 with *Peanut stunt virus* (PSV) as outgroup [31]. The evolutionary history was inferred using the maximum parsimony (MP) method [8]. Tree #1 out of 5 most parsimonious trees (length = 2,393) is shown. The consistency index is 0.477033, the retention index is 0.851434, and the composite index is 0.462543 (0.406163) for all sites and parsimony-informative sites (in parentheses). The MP tree was obtained using the Close-Neighbor-Interchange algorithm [11] with search level 3 [11, 31] in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale, with branch lengths calculated using the average pathway method [11] and are in the units of the number of changes over the whole sequence. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 1,998 positions in the final dataset, out of which 712 were parsimony informative.

Subgroup Specific Detection of CMV

Two pairs of primers, BM53F and BM54R specific for subgroup-I and BM51F and BM52R specific for subgroup-II were designed based on the multiple alignment of full length sequences of RNA-3 of the isolates from both the subgroups. Subgroup specific detection of CMV was conducted by RT-PCR as described previously using an annealing temperature of 54°C .

To check the specificity of these primers for the group specific detection of CMV, was conducted by RT-PCR using experimental samples of tomato plants inoculated

Fig. 1 RNA-3 genome of CMV showing locations of primers used for amplification. *MP* movement protein ORF, *CP* coat protein ORF, *UTR* untranslated region. Arrows indicate location of primers listed in Table 2

Table 3 Percent identity of Tss-In and Tfr-In isolates of CMV with the other isolates reported worldwide based on RNA-3 genome

S. no.	Strain/isolate	Accession	Host	Origin (country and year)	Subgroup	% Identity ^a		CMV Tfr-In				CMV Tss-In			
						RNA-3	MP	IR	CP	RNA-3	MP	IR	CP	RNA-3	MP
1	Q-A	M21464	–	Australia, 1988	II	71.8	79.5	58.1	77.4	95.9	98.5	93.2	98.3	95.9	98.5
2	Ly-A	AF198103	–	Australia, 1999	II	71.7	79.4	57.0	76.8	95.7	99.0	91.7	98.1	95.7	99.0
3	Xb-C	AF268598	Banana	China, 2000	II	72.4	79.9	59.1	77.2	94.2	98.4	90.6	97.1	94.2	98.4
4	PE-C	AF268597	Passion flower	China, 2000	IB	93.2	94.5	85.0	94.8	69.4	77.9	58.3	75.6	69.4	77.9
5	M-C	AF268599	–	China, 2000	IA	90.1	93.5	79.9	93.7	70.1	78.0	58.2	76.2	70.1	78.0
6	PNb-C	DQ512476	<i>Pinellia ternata</i>	China, 2006	IC	85.4	90.9	72.0	91.1	70.0	80.1	56.9	76.2	70.0	80.1
7	phy-C	DQ412732	–	China, 2006	IB	95.0	96.7	90.7	96.4	70.4	78.7	57.6	77.4	70.4	78.7
8	pt-C	DQ409209	<i>Pinellia ternata</i>	China, 2006	IC	85.5	90.8	72.3	91.1	69.9	80.0	57.2	76.2	69.9	80.0
9	BX-C	DQ399550	<i>Pinellia ternata</i>	China, 2006	IC	85.9	90.1	71.7	92.0	70.4	79.5	57.8	76.9	70.4	79.5
10	Tsh-C	EF202597	Tomato	China, 2006	II	72.4	79.5	59.7	77.2	95.5	98.6	91.1	98.3	95.5	98.6
11	cb7-C	EF216867	Tomato	China, 2007	IB	95.4	97.1	91.3	96.4	70.6	78.8	57.3	77.7	70.6	78.8
12	YN-C	EF216865	Brassica sps	China, 2007	IB	95.2	96.7	91.0	96.9	70.6	78.8	57.9	77.7	70.6	78.8
13	CTL-C	EF213025	<i>Brassica chinensis</i>	China, 2007	IB	92.5	94.6	84.2	93.1	69.6	77.4	58.1	76.8	69.6	77.4
14	cah1-C	FJ268746	Canna	China, 2008	IB	95.2	96.9	91.0	96.0	70.4	78.5	57.0	77.7	70.4	78.5
15	PHz-C	EU723569	<i>Pinellia ternata</i>	China, 2008	IC	85.7	90.9	72.3	91.4	70.1	80.1	57.2	76.3	70.1	80.1
16	Te-C	EU665002	Marigold	China, 2008	II	72.3	79.9	59.8	77.2	94.7	98.5	91.9	97.8	94.7	98.5
17	Mb-C	GU002300	<i>Musa basjoo</i>	China, 2009	II	71.2	79.9	60.4	77.1	94.4	98.0	90.6	97.2	94.4	98.0
18	Trk7-H	L15336	–	Hungary, 1994	II	71.9	79.4	59.5	76.3	95.1	99.0	89.6	97.4	95.1	99.0
19	Li-In	AJ831578	Lily	India, 2005	IA	80.5	70.8	77.0	93.4	63.5	60.1	58.5	76.5	63.5	60.1
20	Mp-In	EF178298	Banana	India, 2006	IB	92.2	94.2	86.8	93.1	70.3	78.7	57.1	76.5	70.3	78.7
21	Cm-In	EF153733	Chrysanthemum	India, 2006	IB	92.1	93.6	87.8	91.3	69.7	78.7	56.0	75.3	69.7	78.7
22	Tfr-In*	JF279606	Tomato fruit	India, 2005	IB	100	100	100	100	70.9	79.1	58.8	77.8	70.9	79.1
23	Ts-In	EF153734	Tomato	India, 2007	IB	97.5	97.4	90.0	98.7	70.2	77.6	55.0	77.7	70.2	77.6
24	Jc-In	EF593026	<i>Jatropha curcus</i>	India, 2007	IB	92.3	78.7	87.8	92.0	70.1	93.6	56.0	76.8	70.1	93.6
25	Rs-In	EF593025	<i>Rauvolfia serpentina</i>	India, 2007	IB	92.4	93.5	87.8	92.6	70.1	78.6	56.0	76.6	70.1	78.6
26	Di-In	EF593024	<i>Datura innoxia</i>	India, 2007	IB	92.0	92.9	87.8	91.9	69.7	78.2	56.0	75.9	69.7	78.2
27	At-In	EF593023	Amaranthus	India, 2007	IB	92.0	93.3	87.8	91.6	69.7	78.4	56.0	75.6	69.7	78.4
28	Tss-In*	JF279605	Tomato	India, 2008	II	70.9	79.1	58.8	77.8	100	100	100	100	100	100
29	De-In	EU642567	Carrot	India, 2008	II	71.3	78.2	59.4	76.0	94.6	97.5	91.6	95.7	94.6	97.5
30	NDI-In	GU111229	Tomato	India, 2009	IB	96.1	93.5	89.6	99.2	69.4	74.9	57.1	78.0	69.4	74.9
31	B2-Ia	AB046951	Banana	Indonesia, 2000	IB	92.2	93.8	88.7	93.4	70.0	77.4	56.2	78.1	70.0	77.4
32	IA-Ia	AB042294	–	Indonesia, 2000	IB	92.2	94.1	85.8	92.6	70.1	78.7	57.1	77.1	70.1	78.7
33	Tfr-It	Y16926	Tomato	Italy, 1998	IB	97.4	97.5	90.6	98.7	70.2	78.0	55.1	78.0	70.2	78.0
34	Y1-J	D12499	<i>N. tabacum</i>	Japan, 1991	IA	90.3	94.0	79.6	95.1	70.2	78.4	57.9	77.1	70.2	78.4
35	E5-J	D42080	–	Japan, 1994	IA	90.4	94.4	79.6	94.3	70.2	78.1	58.1	76.3	70.2	78.1

Table 3 continued

S. no.	Strain/isolate	Accession	Host	Origin (country and year)	Subgroup	% Identity ^a		CMV Tfr-In				CMV Tss-In					
								RNA-3		MP	IR	CP		RNA-3	MP	IR	CP
36	C7-2-J	D42079	–	Japan, 1994	IA	89.6	93.5	81.8	94.2	69.3	78.2	58.4	77.2				
37	CS-J	D28489	<i>Limonium sinuatum</i>	Japan, 1994	IA	90.0	93.2	79.3	94.9	71.0	79.1	58.7	77.7				
38	Pepo1-J	D28488	Pepo	Japan, 1994	IA	90.2	93.6	79.6	94.6	70.7	78.4	58.8	77.8				
39	FT-J	D28487	Tomato	Japan, 1994	IA	90.1	93.5	79.3	93.9	70.8	78.2	58.7	77.5				
40	N-J	D28486	<i>Limonium sinuatum</i>	Japan, 1994	IA	90.5	93.6	79.6	95.4	70.4	78.6	58.2	78.0				
41	Y2-J	D83958	–	Japan, 1996	IA	89.9	94.1	79.6	94.5	70.0	78.5	57.9	76.8				
42	MY17-J	AF103993	–	Japan, 1998	IA	90.1	94.4	79.9	94.2	70.4	78.2	58.1	76.9				
43	SO-J	AF103992	–	Japan, 1998	IA	90.4	93.8	79.3	94.5	71.0	78.8	58.4	77.5				
44	Pepo2-J	AF103991	Pepo	Japan, 1998	IA	90.4	93.9	78.6	93.9	71.0	78.5	58.7	77.4				
45	IN-J	AB042294	–	Japan, 2000	IB	92.5	94.1	85.8	92.6	70.3	78.7	58.3	77.1				
46	TN-J	AB176847	Tomato	Japan, 2004	II	72.4	79.8	60.0	76.8	95.9	99.1	91.1	98.1				
47	MT-J	AB189917	Tomato	Japan, 2004	II	72.1	79.7	58.8	77.1	95.5	98.6	91.3	97.5				
48	ON-J	AB248752	Bitter gourd	Japan, 2006	IA	90.4	93.5	79.3	94.9	70.7	78.7	58.7	77.4				
49	PF-J	AB368501	Tomato	Japan, 2007	II	72.2	79.2	60.0	77.5	95.4	98.4	90.4	98.0				
50	Y3-J	AB368498	Cucumber	Japan, 2007	IA	89.6	93.2	79.9	95.1	70.6	78.6	58.7	77.5				
50	cap1-K	DQ777747	Chilli	Korea, 2006	IB	90.8	93.8	78.1	92.6	70.1	77.8	56.0	76.3				
52	Cs-N	AY429437	Groundnut	Netherlands, 2003	IB	90.6	93.5	78.7	92.9	69.7	76.9	54.8	78.3				
53	Ca-N	AY429432	Groundnut	Netherlands, 2003	IB	91.1	93.8	79.1	93.4	69.9	77.3	55.6	78.0				
54	Ix-P	U20219	Tomato	Philippines, 1995	IB	91.2	94.1	82.1	92.9	68.5	76.5	54.4	76.3				
55	Mf-Sk	AJ276481	–	South Korea, 2000	IA	90.7	94.6	79.3	94.9	70.7	78.4	58.7	77.2				
56	Li-Sk	AJ495841	Lily	South Korea, 2002	IA	89.5	94.6	77.7	93.7	70.0	77.9	58.8	76.6				
57	p-Sk	AB369272	Pepper	South Korea, 2007	IA	90.8	94.0	79.9	94.9	70.2	77.9	58.4	76.5				
58	pepY-Sk	AB369271	<i>N. benthamiana</i>	South Korea, 2007	IA	90.6	93.6	79.6	95.1	70.8	79.0	59.0	77.5				
59	V-Sk	AB369270	<i>N. benthamiana</i>	South Korea, 2007	IA	90.2	93.3	78.9	93.7	70.5	78.4	58.1	76.8				
60	Z-Sk	AB369269	<i>N. benthamiana</i>	South Korea, 2007	IA	90.8	93.9	79.3	95.4	70.9	79.1	58.4	77.4				
61	paf-Sk	AB369273	<i>N. benthamiana</i>	South Korea, 2007	IA	90.3	94.2	79.9	94.8	70.1	77.2	58.7	76.8				
62	R18-S	AM183119	Tomato	Spain, 2006	IA	89.5	94.0	79.9	94.6	70.2	78.2	58.4	76.9				
63	pl1-S	AM183116	Tomato	Spain, 2006	IB	96.7	97.0	90.6	98.1	70.4	78.0	56.0	77.8				
64	M48-T	D49496	–	Taiwan, 1995	IB	90.1	93.8	78.9	91.6	70.0	78.4	58.1	76.2				
65	M-U	D10539	–	USA, 1992	IA	90.0	93.6	79.9	93.4	70.1	78.1	58.4	75.9				
66	Fny-U	NC_001440	Summer squash	USA, 1992	IA	90.6	94.0	80.2	94.9	70.2	78.1	58.1	76.8				
67	Ls-U	AF127976	–	USA, 1999	II	71.6	79.3	58.1	77.1	95.9	98.8	93.2	98.1				

^a RNA-3 and IR (intergenic region) are based on nucleotide sequence. MP (movement protein) and CP (coat protein) are based on amino acid sequence. Subgroups are based on sequence identity in complete RNA-3

* Present study

Fig. 2 Symptoms of CMV isolates. **a** Tomato fruit (Tfr) showing grey patches from where Tfr-In isolate was obtained. **b** Tomato plant showing shoe-string (Tss) symptoms from where Tss-In was isolated.

c RT-PCR using primers (BM05F and BM06R) based on CP gene shows detection of CMV in samples from **a** (lane 1) and **b** (lane 2)

with Tfr-In isolate (subgroup-I) and Tss-In isolate (subgroup-II) in the greenhouse. Further, group-specific detection was conducted in field samples of tomato from IARI, New Delhi, and cucurbit and chilli from Bangalore.

Results

CMV Isolates and Host Reactions

A total of five isolates were obtained from cucumber, muskmelon, tobacco and tomato originating from northern, southern and central-western parts of India (Table 1). Tfr-In was isolated from tomato fruit showing grey patches (Fig. 2a) in commercial field at Aurangabad. Another tomato isolate, Tss-In showed shoe-string (Fig. 2b), a distinct symptom-type in the experimental field at IARI, New Delhi. Three other isolates Ban-In, Pun-In and Bal-In showed mottling or mosaic symptoms on tobacco, cucumber and muskmelon, respectively under field conditions. Under greenhouse conditions following sap transmission, Tfr-In, Ban-In, Pun-In and Bal-In isolates produced chlorotic spots and mottling symptoms on tomato, whereas, Tss-In isolate produced shoe-string symptoms. On cucumber, all the isolates produced mosaic mottling and on *N. glutinosa*, severe mosaic blistering symptoms following sap transmission. Host reactions of most of the isolates were similar except Tss-In isolate, which produced distinct symptoms on tomato.

Genome Organization and Phylogenetic Relationships

RT-PCR using CP gene primers, BM05F and BM06R resulted in amplification of 660 bp DNA fragments in all the five isolates. Sequence analysis of complete CP of these isolates showed that four isolates, Tfr-In, Ban-In, Pun-In and Bal-In (JF279606, JF279607, JF279608, JF279609) were closely related with 93–98% sequence identity, whereas, Tss-In showed significant sequence diversity

sharing only 77% identity with the other four isolates. The complete nucleotide sequence of RNA-3 of Tfr-In and Tss-In isolates showed that they were 2,214 and 2,179 nucleotides (nt) long, respectively. The RNA-3 genome contained 5' UTR (Tss-In: 1–63 nt; Tfr-In: 1–111 nt) followed by ORF 3a (Tss-In: 64–903 nt; Tfr-In: 112–951 nt) encoding putative MP, IR (Tss-In: 904–1,199 nt; Tfr-In: 952–1,255 nt), ORF 3b (Tss-In: 1,200–1,856 nt; Tfr-In: 1,256–1,912 nt) encoding CP and 3' UTR (Tss-In: 1,857–2,178 nt; Tfr-In: 1,913–2,214 nt). The RNA-3 genome organization of Tfr-In and Tss-In was similar to other isolates of CMV.

The complete RNA-3 sequence comparison of these two isolates with 65 other isolates of CMV reported world over revealed that Tfr-In was very closely related to subgroup-I isolates sharing a sequence identity of 80.5–97.5%, whereas, it was distantly related to the members under subgroup-II sharing only 70.9–72.4% sequence identity. Interestingly Tss-In isolate was closely related to the members under the CMV subgroup-II sharing of 94.2–95.9% sequence identity and distantly related with the members under subgroup-I sharing only 68.5–71% sequence identity. Tfr-In shared only 70.9% sequence identity with Tss-In in RNA-3. The CP and MP of these two isolates shared 77.8 and 79.1%, respectively and IR was more diverse sharing only 58.8% sequence identity. Comparison of IR sequence of tomato isolates showed that subgroup-II isolates contain sequence diversity at 3' end of IR, whereas majority of subgroup-I isolates showed variability in both termini but three isolates (FT-J, Ix-P and Ri8-S) showed variability throughout the IR (Fig. 4). Phylogenetic analysis based on complete RNA-3 sequence of 67 isolates showed four distinct evolutionary subgroups-IA, IB, IC and II (Fig. 3). Tfr-In isolate clustered with the four tomato isolates from India, Italy and Spain under the subgroup-IB whereas, Tss-In isolate clustered with four other tomato isolates from Japan and China with the members under subgroup-II.

Fig. 3 Parsimonious tree based on nucleotide sequence of complete RNA-3 genome of CMV isolates showing evolutionary clustering of Indian isolates with globally distributed isolates. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level in which the initial trees were obtained with the random addition of sequences (10 replicates). The tree is drawn to scale, with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence. Phylogenetic analyses were conducted in MEGA4. Isolates description as in Table 3

Subgroup Specific Detection

As shown in the Fig. 5a, the RT-PCR using subgroup-I specific primers, BM53F and BM54R resulted in specific amplification of ~386 bp with Tfr-In isolate and no amplification with Tss-In isolate, whereas subgroup-II specific primers, BM51F and BM52R gave a specific amplification of ~376 bp with Tss-In isolate. Whereas, the common primers, BM05F and BM06R resulted in amplification of both Tfr-In and Tss-In isolates (Fig. 2c). Fourteen field samples were tested by using both the subgroup specific primers, of which two out of five tomato samples collected from experimental field of IARI, New Delhi showed specific amplification with only subgroup-II primers (Fig. 5b). The cucurbit samples (muskmelon, cucumber, snake gourd and bitter gourd) collected from Bangalore did not show any specific amplification with both the subgroup-I and -II primers. Whereas, the chilli samples from Bangalore showed amplification with subgroup-I specific primers but not with subgroup-II specific primers (Fig. 5c).

Discussion

The five isolates of CMV collected from different crops and geographical locations in India were studied for their host reactions and CP gene variability. Among these isolates, two distinct tomato isolates, Tfr-In and Tss-In were characterised based on complete sequence of RNA-3 genome. The sequence information was utilized to develop RT-PCR based subgroup specific diagnosis of CMV.

The full length RNA-3 of Tfr-In is 35 nucleotides longer than that of Tss-In, and their sequence were significantly different (70.9% similarity). The IR of the two isolates was more heterogeneous compared to the other part of the genome, the multiple alignment of both the isolates showed maximum difference at beginning (70 nucleotides) and at the terminal (100 nucleotides) portion of the IR. The complete RNA-3 sequence of Tss-In and Tfr-In were compared with 65 CMV isolates which were reported from 14 different countries and 25 plant species during last 22 years. Phylogenetic analysis of these isolate based on

Fig. 4 Nucleotide sequence alignment of IRs of Tfr-In with subgroup-I and Tss-In with subgroup-II tomato isolates of CMV reported from Asia and Europe. Isolate designations are in Table 3

complete RNA-3 showed two major subgroup-I and -II. The subgroup-I was further divided into three major subclades-IA, IB and IC. Similar clustering pattern of these isolates was observed based on CP sequence (data not shown). Several isolates of CMV have been reported from several countries in the world and they are classified into three subgroups, -IA, -IB and -II based on phylogenetic analysis of CP gene and 5' UTR sequence [20]. The phylogenetic analysis revealed an additional subclade IC within the subgroup-I was found to contain four isolates (pH2-C, pNb-C, pt-C and BX-C) reported from *Pinellia ternata* from China during 2006–2008 [34]. CMV is a common viral pathogen in tomato. Complete RNA-3 sequence of 14 isolates of CMV from tomato are available from China, Japan, India, Philippines, Italy and Spain, and they are distributed in both subgroup-I and -II.

The majority of CMV isolates in India are grouped under subgroup-IB. Interestingly, Tfr-In and other tomato isolates from India clustered together under subgroup-IB. Under subgroup-II, Tss-In clustered together with six other tomato isolates reported from Hungary, Japan and China.

Shoe-string symptoms caused by CMV has been reported from India, which was grouped under subgroup-IB [14], whereas CMV isolated from tomato showing shoe-string in Pakistan [2] and Australia [30] were grouped under subgroup-IA and -IB, respectively. In the present study, CMV isolate showing shoe-string in tomato (Tss-In) is grouped under subgroup-II. It seems shoe-string symptom phenotype is not consistent with phylogenetic grouping. Several studies showed subgroup-IB type of CMV is predominant in India [10, 14, 16, 23, 27]. However, only a few Indian isolates were reported under subgroup-II. Subgroup-II CMV causing

Fig. 5 Subgroup specific detection of CMV by RT-PCR.

a Standard experimental samples from greenhouse by using both subgroup-I and subgroup-II primers. **b** Field samples by using subgroup-II primers (BM51F and BM52R) **(c)** with subgroup-I primers (BM53F and BM54R). *M* 1 kb ladder, *lane 1* Tfr-In showing –ve amplification, *lane 2* Tss-In isolate showing +ve amplification, *lanes 3–7* tomato field samples, *lanes 8–13* cucurbit field samples (*lanes 8, 9 and 10* muskmelon; *lane 11* cucumber; *lane 12* snakegourd; *lane 13* bitter gourd), *lanes 14–16* chilli field samples

severe mosaic and stunting in tomato was reported from southern India based on *MspI* restriction site on CP gene [29]. Genome sequence information of this isolate is not available. The present study for the first time described complete RNA-3 genome sequence of a CMV strain under subgroup-II causing shoe-string symptoms in tomato in India. In addition to tomato, subgroup-II isolates were also reported in India infecting carrot (EU642567), lily [25] and geranium [32].

Previously, RT-PCR based detection of CMV subgroup-I and -II was achieved based on *MspI* restriction pattern [18, 26] and immunocapture RT-PCR [35] using CP gene. In the present study, molecular detection by RT-PCR was developed to detect CMV subgroups in Indian population of CMV. The complete sequence comparison of Tfr-In and Tss-In revealed maximum sequence diversity in the flanking IR regions that include the 3' region of MP ORF and 5' region of CP ORF. Therefore, subgroup specific primers were designed based on sequence from the above mentioned regions and specific amplification of

Tfr-In and Tss-In representing subgroup-I and subgroup-II, respectively, was obtained. Further, the specificity of detection was verified by using field samples from different crops.

CMV is known to cause severe damage in tomato crops worldwide showing different types of symptoms like shoe-string, mosaic, necrosis, fruit necrosis and stunting. CMV has been reported to cause fruit necrosis in tomato in Hungary [22]. In Spain, subgroup-IB CMV causes symptomless leaves and discolouration of fruit [3]. In the present study, for the first time a subgroup-IB CMV causing grey patches on tomato fruit has been described and complete RNA-3 genome sequence has been characterized from India. Characterisation of another distinct strain of CMV causing shoe-string in the present study and severe mosaic in the previous study [29] provided evidence that tomato in India is affected by multiple strains of CMV.

Acknowledgments The research was supported by the Department of Biotechnology, Government of India.

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